AMENDMENTS TO THE SPECIFICATION

Please amend the specification at page 8, paragraph [0010] as follows:

It was shown that bacteria could be lysed completely, by removing large food scraps by passing the culture solution through a 5 μm-filter, adding a lytic enzyme solution (mixed solution of Achromopeptidase and lysozyme), treating the mixture at 37°C for 1 h, and adding a mixed solution of surfactant Polysorbate 20 (full name Polyoxyethylene (20) sorbitan monolaurate - commercially known as TWEEN® 20) ~~(Tween 20)~~ and protein denaturant (Guanidine isothiocyanate). Moreover, it was shown that bacteria could be also lysed completely by using bacteriocin having lytic activity such as Enterolysine instead of Achromopeptidase. DNAs were extracted by removing insoluble fractions by centrifugation, and performing alcohol precipitation. It was shown that the following treatment order was extremely preferable: performing lysozyme treatment preferably prior to or simultaneously with Achromopeptidase; performing Enterolysine treatment preferably prior to or simultaneously with lysozyme treatment; then performing Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ treatment followed by Guanidine isothiocyanate treatment, or performing Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ treatment and Guanidine isothiocyanate treatment simultaneously. It was further shown that as Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ has high viscosity, it was difficult to add it separately, and thus adding it as a mixture was preferable.

Please amend the specification at page 9, paragraph [0011] as follows:

Further, even phenol or chloroform treatment is not performed, protein being soluble to a level sufficient to be detected by PCR without problem can be removed by alcohol precipitation or according to the added level of DNA extraction solution (2 μl per 50 μl of PCR

reaction solution). As Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ is also used in PCR reaction solution, there is less inhibition compared to SDS, and thus being easy to be used by unskilled experimenters. Moreover, these extraction methods can be applied for extraction of each bacterium alone, independently. Furthermore, the following methods have been tried: (1) Achromopeptidase alone; (2) lysozyme alone; (3) Enterolysine alone; (4) Achromopeptidase treatment followed by treatment with guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~; (5) lysozyme treatment followed by guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~; (6) Enterolysine treatment followed by treatment with guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~; (7) proteinase K, (8) proteinase K treatment followed by treatment with guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~; (9) guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ treatment; (10) guanidine isothiocyanate + Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ + heating treatment. However, the method comprising treatment with Achromopeptidase and lysozyme in combination, or treatment with Enterolysine and lysozyme in combination, followed by treatment with Polysorbate 20 (TWEEN® 20) ~~Tween 20~~ and guanidine isothiocyanate was excellent from the point of view of Listeria extraction with high sensitivity, compared to any of the above methods.

Please amend the specification at page 17, paragraph [0019] as follows:

In the multiple detection method of the present invention, the process for extracting DNA of contaminating microorganisms in foods which have been cultured and proliferated is indispensable. The DNA extraction process is not particularly limited as long as it is a process for extracting DNA of target microorganisms to be detected, by treating at least with

a lytic enzyme and/or bacteriocin with lytic activity, a surfactant and a protein denaturant, while a method for depositing and extracting DNA by alcohol precipitation after treating with a lytic enzyme and/or bacteriocin with lytic activity, treating with a surfactant and a protein denaturant, and removing insoluble fractions by centrifugation can be preferably exemplified. Examples of the above lytic enzymes include: Achromopeptidase, lysozyme, proteinase K, chitosanase, chitinase, β-1,3-Glucanase, Zymolyase and Cellulase. Examples of bacteriocin having lytic activity include Enterolysine and helveticine. One or more of these can be used, while Achromopeptidase, lysozyme, Enterolysine , or a combination thereof, for example simultaneous use of Achromopeptidase and lysozyme, simultaneous use of Enterolysine and lysozyme, can be preferably exemplified. As for the above surfactants, anion surfactant, cation surfactant, amphoteric surfactant, nonionic surfactant can be exemplified. Among these, ethylene oxide condensate of sorbitan monolaurate which is a nonionic surfactant, more specifically Polysorbate 20 (TWEEN® 20) ~~Tween 20~~, can be preferably exemplified. As for the above protein denaturants, guanidine isothiocyanate, urea, guanidine hydrochloride, trichloroacetate, SDS, Triton X-100 and deoxycholate can be exemplified. One or more of these can be used, while guanidine isothiocyanate is preferable from the view point of bacteriolysis or easy handling. Extract/deposit of DNA from lysates can be performed by commonly known methods such as removing insoluble fractions by centrifugation, and then performing alcohol precipitation.

Please amend the specification at page 22, paragraph [0022] as follows:

As a result, by using a medium with glucose concentration of 0.15% or less, or a medium with phosphate concentration of 50 mM or more, or a medium maintaining the pH after culture at 5.1 or more, all of pathogenic Escherichia coli 0157, Salmonella spp. and Listeria

monocytogenes proliferated to 103 CFU/ml or more (cell counts necessary for detection by PCR) by culturing for 18 h or more. For the tests thereafter, medium No. 17 of Table 1 (tryptose 10 g, meat extract 5 g, yeast extract 5 g, sodium chloride 5 g, glucose 0.5 g, disodium phosphate 7 g, monopotassium phosphate 15 g/l L) was selected. As for medium components, ni trogen sources other than tryptose, meat extract or yeast extract, carbon sources other than glucose, substances with buffer ability other than phosphate are also effective according to the existing environment or damage level of the bacteria to be detected. Further, it was more preferable to add inorganic salts, pyruvic acid or pyruvate salt, or surfactants such as Polysorbate (TWEEN®) ~~Tween~~ as substances promoting proliferation.

Please amend the specification at page 24, paragraph [0028] as follows:

(DNA extraction method 1) Each bacteria of pathogenic Escherichia coli 0157, Salmonella spp. and Listeria monocytogenes were inoculated to 10 ml of medium No. 17, and cultured at 35°C for 18 h. Each culture solution was taken to a 1 ml-tube, respectively, centrifuged at 15,000 r.p.m for 5 min to collect bacteria. Lytic enzyme solution {mixed solution of 10 μl of 20 mg/ml Achromopeptidase and 10 μl of 20 mg/ml Lysozyme, and 180 μl of TE buffer [10 mM Tris (hydroxymethyl) aminomethane-hydrochloric acid buffer solution containing 1 mM EDTA, pH 8]} was added, treated at 37°C for 1 h. Then, 300 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to dissolve bacteria completely. By observing this solution with a light microscope, it was confirmed that lysis was sufficiently performed. The solution was centrifuged at 15.000 r.p.m for 5 min and 400 μl of the supernatant was transferred to another tube. After precipitating DNA in the lysate with isopropanol, the resultant was centrifuged, and thus, the

intended DNA was obtained. Further. by using Enterolysine instead of Achromopeptidase, it was confirmed that lysis was sufficiently performed, similarly.

Please amend the specification at page 25, paragraph [0029] as follows:

(DNA extraction method 2) Similarly, each bacteria of pathogenic Escherichia coli 0157, Salmonella spp. and Listeria monocytogenes were inoculated to 10 ml of medium No. 17. and cultured at 35°C for 18 h. Each culture solution was taken to a 1 ml-tube, respectively, centrifuged at 15.000 r.p.m for 5 min to collect bacteria. 500 μl of lytic agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to the collected bacteria to dissolve collected substances. The resultant solution was heated at 100°C for 10 min. and ice cooled for 5 min. By observing this solution with a light microscope. it was confirmed that pathogenic Escherichia coli 0157 and Salmonella spp. were lysed, while the lysis level of Listeria monocytogenes was a little lower compared with that of the DNA extraction method of Example 3. Further, lysis of Listeria monocytogenes lysate was performed by adding the following: 1) a solution to which a mixed solution of 10 μl of 20 mg/ml Achromopeptidase and 190 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 2) a solution to which a mixed solution of 10 μl of 20 mg/ml of Lysozyme and 190 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 3) a solution to which a mixed solution of 10 μl of Enterolysine and 190 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 4) a solution to which 300 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to the above 1) solution and mixed; 5) a solution to which 300 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20

(TWEEN® 20) ~~Tween 20~~) was added to the above 2) solution and mixed; 6) a solution to which 300 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to the above 3) solution and mixed; 7) a solution to which a mixed solution of 1 μl of 20 mg/ml ~~proteinaze~~ proteinase K1 and 200 μl of TE buffer was added to the collected bacteria, and treated at 37°C for 1 h; 8) a solution to which 300 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to the above 7) solution and mixed; 9) a solution to which 500 μl of lysing agent (4 M guanidine isothiocyanate solution supplemented with 1 to 2% of Polysorbate 20 (TWEEN® 20) ~~Tween 20~~) was added to the collected bacteria and mixed. The resultants were observed with a light microscope. For all of these, the lysis level of Listeria monocytogenes was lower compared with that of DNA extraction method of Example 3.

Please amend the specification at page 34, paragraph [0038] as follows:

As for pathogenic Escherichia coli 0157, 225 ml of novobiocin mEC broth (Kyokuto Pharmaceutical Industrial) was added to 25 g of sample, which was ground with a stomacher for 30 sec, cultured at 42°C for 18 hours, streaked in CHROMagar 0157 medium (Kanto Chemical) and MacConkey Sorbitol Agar medium (Nissui Pharmaceutical), and cultured at 35°C for 18 to 24 h. Those who showed lilac in CHROMagar 0157 medium, and translucent pink in MacConkey Sorbitol Agar medium were determined to be false-positive pathogenic Escherichia coli 0157, and were streaked in CLIG agar medium (Kyokuto Pharmaceutical Industrial) and cultured at 35°C for 18 to 24 h. Those whose lower layer was yellow and upper layer was pink, and that were not luminescent to ultraviolet radiation were determined to be

false-positive pathogenic Escherichia coli 0157. They were subjected to indole reaction, and for those being positive (red) , agglutination reaction was performed. Agglutination reaction was performed by using the Escherichia coli 0157 detection kit known as "UNI® kit" (Oxoid). Colonies that were doubtful by agglutination reaction were streaked in CHROMagar 0157 medium, MacConkey Sorbitol agar medium, TSI agar medium (Nissui Pharmaceutical) and LIM medium (Nissui Pharmaceutical), and were cultured at 35°C for 24 hours. Those who showed lilac in CHROMagar 0157 medium, translucent pink in MacConkey Sorbitol agar medium, yellow in TSI agar medium and showing no change in LIM medium, were confirmed to be pathogenic Escherichia coli 0157 by PCR reaction.

Please amend the ABSTRACT as follows:

The present invention is to provide a multiple detection method that can detect contaminating microorganisms existing in foods, including pathogenic Escherichia coli O157, Listeria monocytogenes and Salmonella spp., with high sensitivity comparable or even superior to official methods, comprising the steps of amplifying a plural number of target genes with a single PCR reaction tube and analyzing the same. The following steps are performed consecutively: (A) a step of extracting DNA of the target microorganisms to be detected by treating with at least a lytic enzyme such as Achromopepidase and Lysozyme ~~Lyzocyme~~ and/or bacteriocin having lytic activity such as Enterolysine, a surfactant and a protein denaturing agent; and (B) a step of mixing a specific primer to the target microorganisms to be detected to perform multiplex PCR. Further, it is preferable to add a step of culturing with a culture condition where 1 CFU/100 g microorganisms becomes 10.sup.3 CFU/ml or more after 18 to 48 h of culture, for

example that the pH after culture becomes 5.1 or more, before the step of extracting DNA of the target microorganisms to be detected.